MULTI-TARGETED NITROGEN BEARING HETEROCYCLES FOR ALZHEIMER'S DISEASE

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Multi-Targeted Nitrogen Bearing Heterocycles for Alzheimer's Disease

Introduction: Alzheimer's disease (AD) is a progressive neurodegenerative disorder accounting for nearly 70% of dementia cases worldwide. It affects over 55 million people globally, with projections reaching 139 million by 2050, especially impacting low- and middle-income countries like India. AD is characterized by neuronal death, neurofibrillary tangles, amyloid plaques, and cerebral amyloid angiopathy. Tau hyperphosphorylation, driven by dysregulated kinases such as GSK-3β and CK-1δ, plays a central role in disease progression. These kinases disrupt Wnt/β-catenin signaling and contribute to neuroinflammation and amyloid pathology.

Due to the multifactorial nature of AD, a multitarget-directed ligand (MTDL) strategy is promising. Dual inhibition of GSK-3β and CK-1δ may effectively reduce tau pathology. This study focuses on designing nitrogen-containing heterocycles as dual kinase inhibitors. The work includes their synthesis and biological evaluation. The goal is to develop safe and effective anti-Alzheimer's agents with neuroprotective potential.

Objectives:

1) Exploring the potential of GSK-3β and CK-1δ inhibitors in the treatment of multifaceted Alzheimer Disease.
2) To carry out *in silico* study for the designing and identification of potent dual GSK-3β and CK-1δ inhibitors.
3) Synthesis of designed analogues and their characterization.
4) To perform the biological activity of the synthesized compounds.

Methodology (work done):

Designing: The synthesis schemes were designed based on the pharmacophore obtained through virtual screening of public domain databases includes Molecular Diversity Preservation International (MDPI)

database, SPEC the Drug Bank database, knowledge-based (KB) database and the National Cancer Institute (NCI).

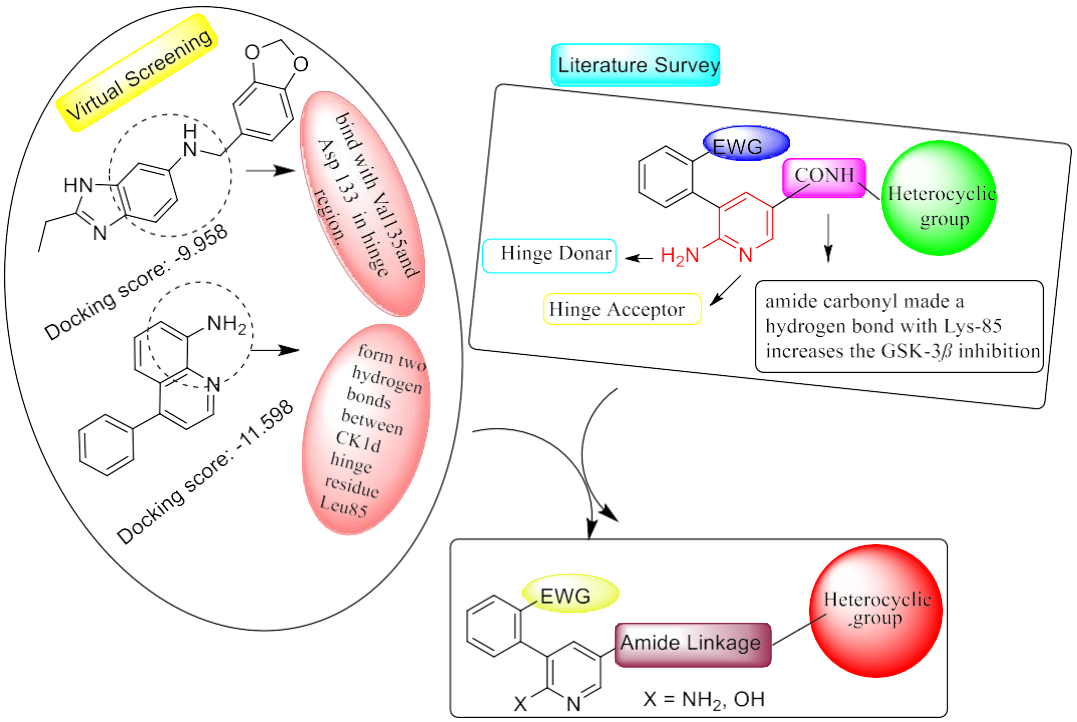
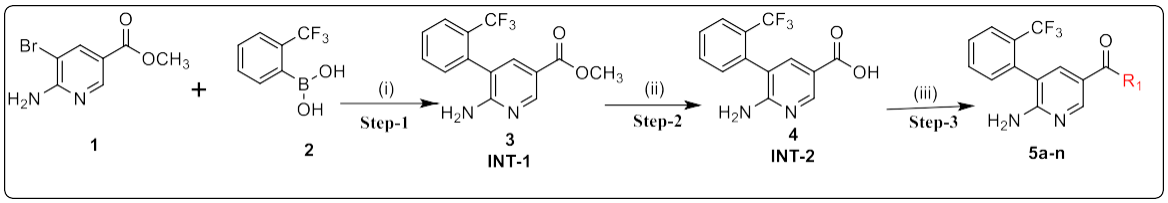
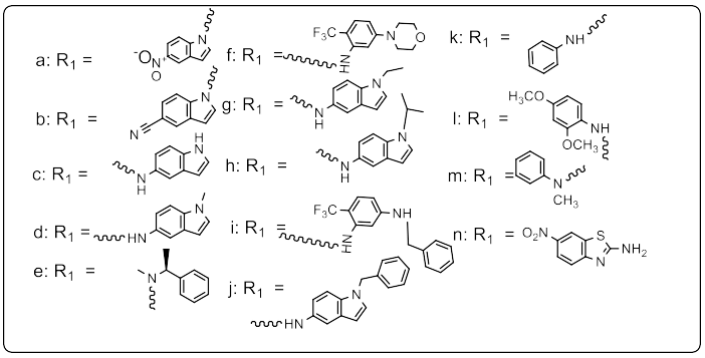


Figure 1. Designed Pharmacophore for Dual Inhibition of GSK-3β and CK-1δ

Synthetic study: Several synthetic schemes were designed, but only three were successfully implemented to synthesize the desired compounds. The reactions were monitored on silica TLC plates to ensure completion of the starting material, and the purification was achieved using 60-400 mesh size silica-gel column chromatography to obtain the desired products.

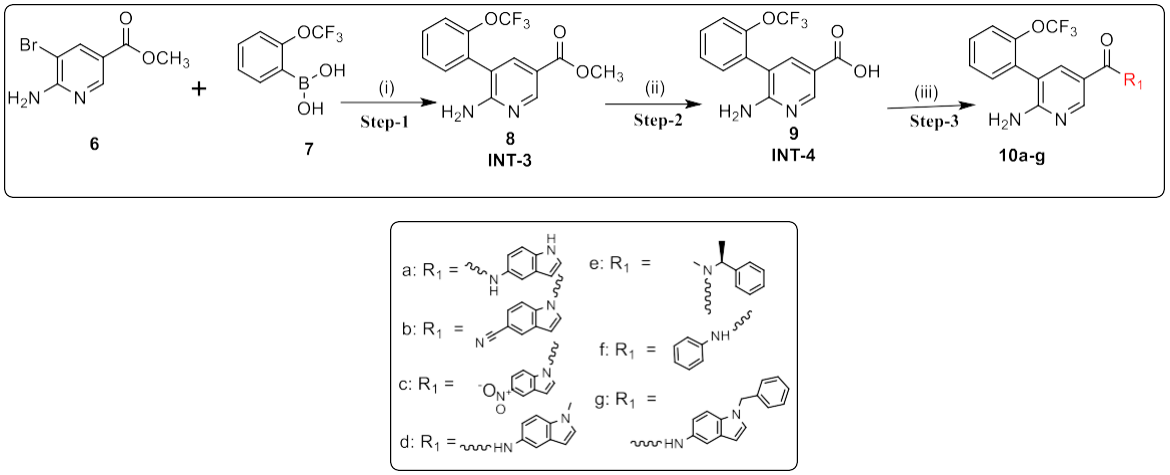
Synthetic scheme-1





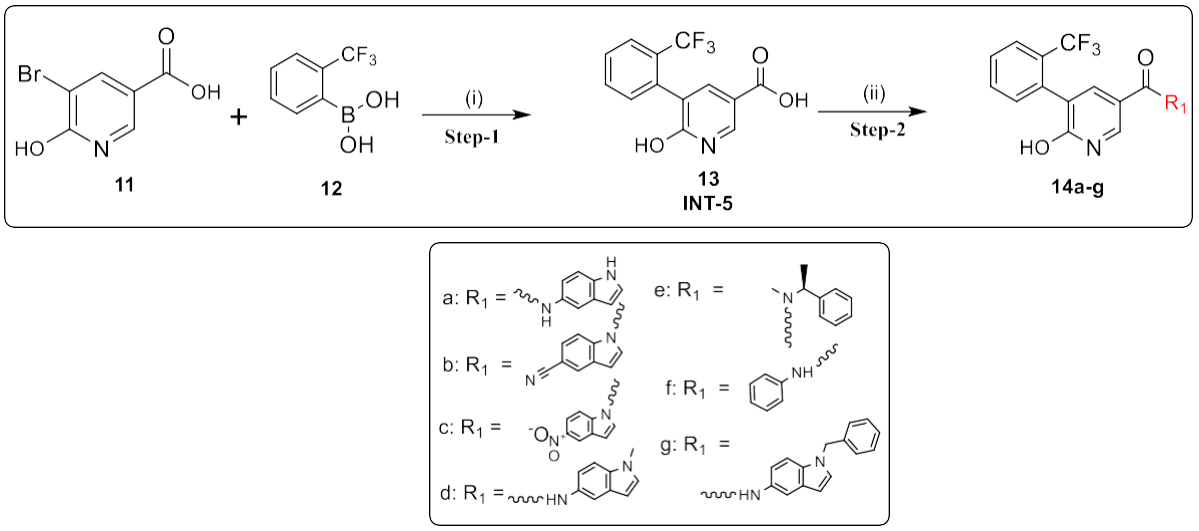
Scheme I: *Reagents and conditions*: i) K_2CO_3/H_2O $PdCl_2(dppf)$, THF, μw, 120°C, 60 min. ii) $Li(OH)_2$ THF/CH_3OH/H_2O (5:1:1), RT, 16h, iii) Substituted amines, HATU, DIPEA/DMF, 16h, RT

Synthetic scheme-2



Scheme 2: *Reagents and conditions*: i) K_2CO_3/H_2O $PdCl_2(dppf)$, THF, μw, 120°C, 60 min. ii) $Li(OH)_2$ $THF/CH_3OH/H_2O$ (5:1:1), RT, 16h, iii) Substituted amines, HATU, DIPEA/DMF, 16h, RT

Synthetic scheme-3



Scheme 3: *Reagents and conditions*: i) K_2CO_3/H_2O $PdCl_2(dppf)$, Dioxane, μw, 120°C, 60 min. ii) Substituted amines, HATU, DIPEA/DMF, 16h, RT

Figure 2. (Scheme-I), (Scheme-II) & (Scheme-III)

Structural elucidation: The final synthesized compounds were analyzed using 1H-NMR spectroscopy and further confirmed by mass spectrometry.

Biological evaluation: *Invitro* biological activity was evaluated using Kinase-Glo MAX Luminescence assay kit from BPS Bioscience (Catalog No. V6071) for GSK-

3β and sandwich enzyme immunoassay kit (BPS Bioscience; Catalog No. MBS2024500) for CK-1δ, according to the prescribed protocol

Results:

Designing: Synthesis schemes were designed based on the 6 amino/hydroxy pyridine pharmacophore necessary for the dual inhibition of GSK-3β and CK-1δ is obtained from virtual screening and literature survey is consists of pyridine nucleus with an amide linkage at the third position, clubbed with a heterocyclic group and an amino group at the sixth position, along with phenyl group with an electron withdrawing group at the fifth position.

Synthetic study: Three synthetic schemes were successfully employed to synthesized 28 pyridine derivatives. Among them, 21 were 6-amino pyridine derivatives and 7 were hydroxy derivatives. All the three synthetic strategies consist of two to three steps: Suzuki (C-C coupling), ester hydrolysis and acid amine coupling. The synthesized pyridine derivatives were obtained as semisolid or solid, with percent yields ranging from 25% to 75%.

Structural elucidation: All the synthesized quinoline derivatives were confirmed by identification of their characteristic peaks in the 1H-NMR spectroscopy, which is further confirmed by mass spectrometry.

Molecular docking study: All the synthesized compounds (**5a-n, 10a-g and 14a-g**) were subjected for molecular docking study using GLIDE tool against the targeted protein GSK-3β and CK-1δ having PDB id: 5KPM and 5OKT. The electron-deficient nature of pyridine facilitates favorable electronic interactions within the kinase binding pockets, contributing to stronger binding affinity. The nitrogen atom of the pyridine ring functions as a hydrogen bond acceptor, often forming key interactions with conserved hinge region residues such as Val135 and Asp133 in GSK-3β, and corresponding residues like leu 85 in CK-1δ whereas replacement of amino group of series 5 and 6 with hydroxy group impacts the interaction making the hydroxy group act as hydrogen bond acceptor with key amino acids of GSK-3β and CK-1δ as shown in the figures of **Chapter 5.**

Most of the compounds exhibited favorable docking scores ranging between -4.2 to -7.8 kcal/mol for both GSK-3β and CK-1δ, indicating good binding affinity toward both

targets. Among the synthesized derivatives, compounds **5d, 10d**, and **14d** showed superior docking scores, correlating well with their *in-vitro* IC_{50} values. These compounds demonstrated strong hydrogen bonding interactions with key amino acid residues such as Asp133, Val135, Lys85, and Arg141 in GSK-3β, and Leu85, Glu83, Asp149, and Leu85 in CK-1δ, which are known to be crucial for kinase inhibition.

Biological evaluation:

GSK-3*β* and CK-1*δ* enzyme inhibition study: All the synthesized compounds were initially evaluated through molecular docking studies against both GSK-3*β* and CK-1*δ*. Compounds that demonstrated favorable docking scores and strong binding interactions with these targets were further selected for enzymatic inhibition studies. A total of 20 synthesized compounds were tested for their inhibitory activity against GSK-3*β*, using AR-A014418 as the reference standard. Based on their promising activity in GSK-3*β* inhibition and docking results, five compounds were shortlisted for subsequent evaluation against CK-1*δ*, with PF-670462 serving as the reference inhibitor. The majority of the synthesized compounds exhibited moderate to potent dual inhibitory activity against both GSK-3*β* and CK-1*δ*. Among them, compounds 5d (6-amino-N-(1-methyl-1H-indol-4-yl)-5-(2-(trifluoromethyl) phenyl) nicotinamide), 10d (6-amino-N-(1-methyl-1H-indol-5-yl)-5-(2-(trifluoromethoxy) phenyl) nicotinamide), and 14d (6-hydroxy-N-(1-methyl-1H-indol-5-yl)-5-(2- (trifluoromethyl)phenyl) nicotinamide) exhibited the most potent dual inhibition. The IC_{50} values against GSK-3*β* were 0.775 μM for 5d, 0.90 ± 0.02 μM for 10d, and 0.575 μM for 14d, while the IC_{50} values against CK-1*δ* were 0.851 μM for 5d, 0.91 ± 0.01 μM for 10d, and 0.495 μM for 14d, respectively.

Cytotoxicity study

To verify that the examined compounds are devoid of cytotoxicity, the toxicity induced by certain chemical substances brain cells are investigated. So, the cell viability was then measured using the MTT test to access the safety of compounds **5d, 5e, 10d** and **14d**. The results demonstrated compounds' **5d** and **14d** low cytotoxicity against neuroblastoma (SHSY5Y) (IC_{50} ranges between 0.09 ± 1.21 μM and 0.08± 1.32 μM) cell lines when compared to both the standard (AR-A014418 & PF-670462) having IC_{50} value of (0.08 ± 1.47 μM and 0.08± 0.77 μM) respectively.

Tau Inhibition Studies

The top most active compounds were further subjected for cell viability assay. As previously mentioned, the build-up of hyperphosphorylated tau leads to the formation of NFTs with

subsequent neuronal death. Using **Horseradish Peroxidase-based Enzyme- Linked Immunosorbent Assay kit**. the phosphorylation status of tau and inhibitory effect of **5d and 14d** were assessed at the cellular level. The kit employs an antibody that binds specifically to phosphorylated tau, and this antibody is conjugated to **Horseradish Peroxidase (HRP)**. When tau phosphorylation occurs, the HRP-linked antibody binds to the phosphorylated tau. A substrate is then added, and the HRP enzyme catalyzes a reaction that produces a measurable color change, indicating the presence and quantity of phosphorylated tau. In tau inhibition studies, synthesized compounds are tested to determine their ability to reduce tau phosphorylation. A decrease in the color intensity during the assay indicates effective inhibition of tau phosphorylation by the tested compounds, which is essential for evaluating their therapeutic potential in Alzheimer's disease. This assay provides a quantitative measure of tau inhibition and as the concentration of an inhibitor increases, its ability to reduce tau phosphorylation (inhibit tau protein) is expected to rise, assuming the inhibitor is effective. This is measured by a decrease in the signal (color intensity) generated in the HRP-based assay. Here both the compound **5d** and **14d** showed comparable results (90.97662383 pg/ml and 49.67021073 pg/ml respectively) with the standards AR-A014418 & PF-670462 (116.8312283 pg/ml and 77.66331408 pg/ml).

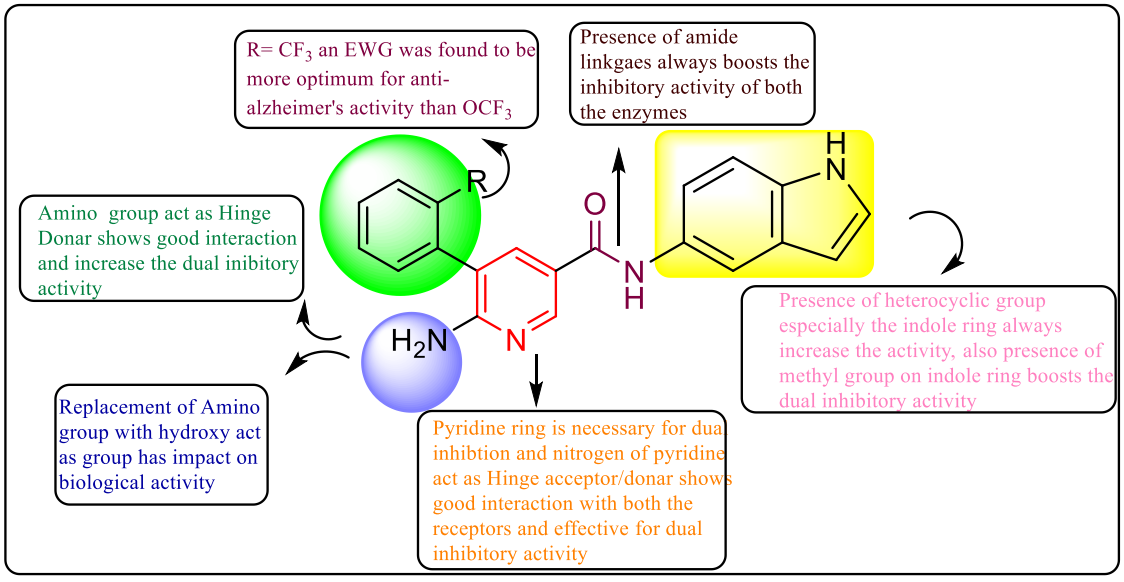

R= CF3 an EWG was found to be more optimum for anti-alzheimer's activity than OCF3
Presence of amide linkgaes always boosts the inhibitory activity of both the enzymes
Amino group act as Hinge Donar shows good interaction and increase the dual inibitory activity
Presence of heterocyclic group especially the indole ring always increase the activity, also presence of methyl group on indole ring boosts the dual inhibitory activity
Replacement of Amino group with hydroxy act as group has impact on biological activity
Pyridine ring is necessary for dua inhibtion and nitrogen of pyridine act as Hinge acceptor/donar shows good interaction with both the receptors and effective for dual inhibitory activity
R
O
N
H
H2N
N
H
N

Figure 3 Comprehensive SAR study

Conclusion

The current research yielded the following significant findings and conclusions:

- In the present study, the key pharmacophoric features essential for dual inhibition of GSK-3β and CK-1δ, involved in tau hyperphosphorylation in Alzheimer's disease, were identified through extensive literature review and

structure-based virtual screening of public domain databases (MDPI, SPEC, DrugBank, KB database, and NCI database).

- Based on the identified structural features, compounds and synthetic schemes were rationally designed. A total of 27 novel pyridine-based derivatives were synthesized through three synthetic schemes and categorized into three series (**5a-n, 10a-g, and 14a-f**). The structures of synthesized compounds were confirmed by spectroscopic characterization using NMR, MS, and FT-IR techniques. Physical properties like melting point and R_f values were also determined for all synthesized compounds.
- Molecular docking studies were performed to investigate the binding interactions of all synthesized compounds with GSK-3β and CK-1δ enzymes. Based on favorable docking scores and strong binding interactions, selected compounds were screened for in vitro enzymatic inhibition studies. A total of 20 compounds were tested for GSK-3β inhibition, using AR-A014418 as reference standard, and the most active compounds were further evaluated for CK-1δ inhibition using PF-670462 as reference.
- Enzymatic inhibition results revealed that compounds **5d, 10d,** and **14d** exhibited potent dual inhibition activity. Specifically, compound **5d** (IC_{50} = 0.775 μM for GSK- 3β; 0.851 μM for CK-1δ), compound **10d** (IC_{50} = 0.90 ± 0.02 μM for GSK-3β; 0.91 ± 0.01 μM for CK-1δ), and compound **14d** (IC_{50} = 0.575 μM for GSK-3β; 0.495 μM for CK-1δ) demonstrated superior inhibitory activity comparable to the reference standards.
- The most potent compounds (**5d, 10d, and 14d**) were further evaluated for cytotoxicity using MTT assay on SH-SY5Y neuronal cells to assess neurotoxicity. All selected compounds exhibited minimal cytotoxicity at therapeutically relevant concentrations, confirming their safety profile for further development as potential neuroprotective agents in Alzheimer's disease.
- The top active compounds were further evaluated for their ability to inhibit tau phosphorylation using HRP-based ELISA assay. Both **5d** and **14d** showed significant reduction in phosphorylated tau levels (90.97 pg/mL and 49.67 pg/mL respectively), comparable to reference standards AR-A014418 and PF-670462.

The findings suggest that the designed pyridine-based dual inhibitors targeting GSK-3β and CK-1δ hold significant promise for further preclinical development as multitarget- directed ligands (MTDLs) for the treatment of Alzheimer's disease by modulating tau pathology. This research has successfully identified and validated a new class of dual GSK-3β/CK-1δ inhibitors based on the 6-amino/hydroxy pyridine scaffold. The lead compounds, particularly **5d** and **14d**, demonstrated a synergistic mechanism of action, combining kinase inhibition with tau phosphorylation suppression and neuroprotective properties. These findings provide a promising platform for further and optimization of multitarget therapeutics for effective intervention in Alzheimer's disease.